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(57) Abstract

A method for myocardial, renal or hepatic opacification comprising the steps of: (a) obtaining an echo contrast agent which comprises: (i) an aqueous albumin-dextrose solution containing between about a two-fold and about an eight-fold dilution of between about 5 % to about 50 % by weight dextose and between about 2 % to about 10 % by weight human serum albumin, and (ii) microbubbles, the gaseous content of which contains an amount of perfluorocarbon gas which is effective for visually detecting myocardial perfusion by echocardiogram following peripheral intravenous injection of said agent into a host; (b) introducing said echo contrast agent into a host by intravenous injection; and (c) performing an echo contrast study on said host using a suitable Doppler or ultrasound echo apparatus.

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ULTRASOUND CONTRAST AGENTS, CONTAINING PERFLUOROCARBON IN DEXTROSE-ALBUMIN MICROBUBBLES

FIELD OF THE INVENTION

This invention relates to a new and improved ultrasonic contrast agent and to its manufacture and use in ultrasonic imaging and echocardiography. More particularly, the contrast agent of this invention relates to the sonicated microbubble type, but is unique in that it makes possible the non-invasive visual detection of myocardial uptake, as discussed more fully hereinafter. In addition, the contrast agent of this invention makes feasible safe and consistent, non-invasive methods for visually assessing, qualitatively or quantitatively, not only myocardial perfusion, but renal and hepatic perfusion, and for detecting severity of coronary arterial stenosis.

BACKGROUND OF THE INVENTION

Ultrasonic imaging is used as a diagnostic tool to aid in therapeutic procedures. It is based on the principle that waves of sound energy can be focused upon an area of interest and reflected to produce an image. Generally, an ultrasonic transducer is placed on a body surface overlying the area to be imaged, and ultrasonic energy, produced by generating and receiving sound waves is transmitted. The ultrasonic energy is reflected back to the transducer where it is translated into an ultrasonic image. The amount and characteristics of the reflected energy depend upon the acoustic properties of the tissues, and contrast agents which are echogenic are preferentially used to create ultrasonic energy in an area of interest and improve the image received.

In ultrasound imaging, videotape images obtained following contrast injection are digitized, allowing the gray scale to be quantified from 1 to 225 gray scale units for 30 cardiac cycles. The contrast intensity is plotted on the vertical axis against time on the horizontal axis.

The peak videointensity (corrected for baseline intensity) is determined as the highest point on the time intensity curve.

For a discussion of contrast echographic instrumentation, see, for example, De Jong N, "Acoustic properties of ultrasound contrast agents", CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAG (1993), pages 120 et seq.

Contrast echocardiography has been used to delineate intracardiac structures, assess valvular competence, and demonstrate intracardiac shunts. Myocardial contrast echocardiography (MCE) has been used to measure coronary blood flow reserve in humans. MCE has been found to be a safe and useful technique for evaluating relative changes in myocardial perfusion and delineating areas at risk.

A multiplicity of potential ultrasonic imaging agents has been reported for contrast echocardiography. No such agent routinely attains visually discernible myocardial uptake following peripheral intravenous injection. Although there have been many reports of transpulmonary transmission of ultrasound contrast agents following intravenous injection and despite the fact that myocardial opacification on echocardiogram can be produced by left sided injection of such contrast agents, visualization of myocardial contrast has not been achieved by intravenous administration of sonicated microbubbles.

Most recently, sonicated albumin and sonicated dextrose/albumin have been shown to produce variable degrees of left ventricular chamber ultrasound contrast following intravenous injection. (See Villanueva et al. Circulation 85:1557-1564, 1992; Lin et al. Int J Card Imaging 8:53-6, 1992; Feinstein et al. J Am Coll Cardiol 16:316-224, 1990; Keller et al. Am Heart J 114:570-575, 1987; and Shapiro et al. J Am Coll Cardiol 16:1603-1607, 1990). The microbubbles of these contrast agents are small (4-6 microns) and are capable of swift transpulmonary passage. However, visually discernible myocardial uptake of such microbubbles following peripheral intravenous

injection has not been possible because of the rapid diffusion of blood soluble oxygen and nitrogen inside the microbubble into the blood which consequently loses its ultrasound reflective properties (e.g., see Porter et al. J Am Soc Echocard Supplement 7:S1, May 1994, and Weyman AE: Principles and Practice of Echocardiography, Malvern, Pennsylvania: Lea & Febiger, 1994; pp. 302-26.)

An important objective of this invention is to provide a contrast agent and methods for its production and use wherein microbubble survival and subsequent myocardial ultrasound contrast is improved sufficiently to make possible visually discernible myocardial uptake of such microbubbles following non-invasive peripheral intravenous injection. This and other objectives of this invention will become apparent in the following discussion.

SUMMARY OF THE INVENTION

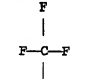
In accordance with this invention, there is provided an improved ultrasound contrast agent which relies on microbubbles for echogenicity, which comprises enhancing the internal atmosphere of the microbubbles with an amount of perfluorocarbon gas which is effective for visually detecting myocardial uptake upon echocardiogram following peripheral intravenous injection of said agent into a host. The perfluorocarbon gas content of the microbubbles is sufficient to lower microbubble gas solubility and diffusivity *in vivo* in blood. Generally, the minimum amount of perfluorocarbon gas in the microbubbles which is effective is that amount which results in microbubbles which pass reliably through the pulmonary circulation without collapse. This is evidenced by opacification of the myocardium of the left ventricle of the heart following intravenous injection and can be visually discerned by echocardiography, for example, in accordance with standard methods or the methods described more fully hereinafter.

Consequently, the invention also provides a method of ultrasonic imaging for use in medical procedures,

comprising the steps of injecting the unique perfluorocarbon-containing microbubbles of this invention into a host to thereby alter the acoustic properties of a predetermined area, and ultrasonically scanning an area including said predetermined area so as to obtain an image of said predetermined area.

DETAILED DESCRIPTION OF THE INVENTION

The perfluorocarbon-enhanced contrast agents of the invention comprise any contrast agent for ultrasonic imaging which relies on microbubbles for ecogenicity the interior of which are enhanced with any insoluble gas such as perfluorocarbon gas. The chemical compound must be a gas at body temperature and be nontoxic. The gas must also form stable microbubbles of average size of between about .1 and about 10 microns in diameter when the contrast agent is sonicated to form microbubbles. Generally the gases are perfluorocarbon gases having the following formula:



wherein n is a whole number integer from 1-10

This includes perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane, perfluoropentane, etc. and other such perhalocarbon gases. Of these gases, perfluoropropane (C_3F_8) and perfluorobutane (C_4F_{10}) are especially preferred because of their demonstrated safety for intraocular injection in humans. They have been used in human studies for intraocular injections to stabilize retinal detachments (Wong and Thompson, Ophthalmology 95:609-613) and are useful in treating complicated retinal detachments by providing internal tamponade of retinal breaks. Treatment with intraocular perfluoropropane is considered to be the standard of care for treatment of this disorder. In a most preferred embodiment the gas is

perfluorobutane, however, it should be apparent to one of ordinary skill in the art that other inert gases such as sulfur hexafluoride are also useful for the invention, provided they have a diffusion coefficient and blood solubility lower than nitrogen or oxygen.

For most ultrasound imaging, the contrast agent is formulated in a pharmaceutically effective dosage form for peripheral administration to the host to be imaged. Generally such host is a human subject although other mammalian hosts, such as canine or equine can be imaged effectively. In a most preferred embodiment the contrast agent is a sonicated mixture of commercially available albumin (human), USP, solution (generally supplied as 5% or a 25%, by weight, sterile aqueous solutions), and commercially available dextrose, USP, for intravenous administration are employed. This mixture is sonicated under ambient conditions, i.e., room air, temperature and pressure, and is perfused with perfluorocarbon or other commercially available inert gas (99.9% by weight) during sonication.

In a preferred embodiment the invention uses a microbubble contrast agent wherein the microbubbles are stabilized by a filmogenic, de-naturable protein coating. Suitable proteins include naturally occurring proteins such as albumin, human gamma-globulin, human apotransferrin, Beta-lactose, and urease. The invention preferably employs a naturally occurring protein, but synthetic proteins may also be used. Particularly preferred is human serum albumin.

Although intravenous echo contrast agents made from sonicated microbubbles are known (e.g., ALBUNEX, Molecular Biosystems, Inc.) and can be employed in this invention, it is preferred to use a sonicated aqueous solution containing a mixture of a pharmaceutically acceptable saccharide, e.g., dextrose, and a protein, e.g., albumin. Generally, sonication is performed in an air atmosphere. In an especially preferred embodiment, dextrose, which is readily

available in pharmaceutically acceptable dosage forms, is the preferred saccharide and human serum albumin is the preferred protein. The preferred embodiment would include a two-fold to eight-fold dilution of 5%-50% by weight of dextrose and a 2%-10% by weight of human serum albumin. Exemplary of other saccharide solutions of this invention are an aqueous monosaccharide solution (e.g. having the formula $C_6H_6O_{12}$, such as, the hexoses, dextrose or fructose, or mixtures thereof), aqueous disaccharide solution (e.g., having the formula $C_{12}H_{22}O_{11}$, such as sucrose, lactose or maltose, or mixture thereof), or aqueous polysaccharide solution (e.g., soluble starches having the formula $(C_6H_{10}O_5)_n$, wherein n is a whole integer between about 20 and about 200, such as amylose or dextran, or mixtures thereof. Sonication by ultrasonic energy causes cavitation within the dextrose-albumin solution at sites of particulate matter or gas in the fluid. These cavitation sites eventually resonate and produce small microbubbles (about 4 to about 7 microns in size) which are non-collapsing and stable. In general, sonication conditions which produce concentrations of greater than about 4×10^8 m of between about 5 and about 6 micron microbubbles are preferred.

The mean microbubble size of sonicated dextrose albumin ranges from between about 5 to about 6 microns. This is a good size as it has been observed that microbubble radius decreases as a function of time in a still liquid due to a diffusion gradient present between the internal and external gases of the microbubble. An increase in microbubble size has a significant effect on the persistence of a microbubble within blood. It must also be of a size sufficient for transpulmonary passage. It must have a mean diameter of less than 10 microns and greater 0.1 microns. Since the size of albumin microbubbles is ideal (between 5 and 6 microns) for transpulmonary passage, the major reason for the significant loss in left ventricular and myocardial

videointensity produced following intravenous injection of albumin coated microbubbles is due to the significant diffusion of gases within the microbubble following intravenous injection during transit to the left ventricular cavity. Sonicated dextrose albumin enhanced with an inert gas such as perfluorocarbon gas, having a lower blood solubility than air, produces a significantly higher left ventricular and myocardial videointensity than sonicated albumin alone.

In addition to myocardial imaging the contrast agents of this invention are useful for renal and hepatic imaging. Thus, another embodiment of this invention provides a method for myocardial, renal or hepatic opacification. The method preferred involves obtaining an echo contrast agent of this invention, introducing said echo contrast agent into a host by intravenous injection, and performing an echo contrast study on said host using a suitable Doppler or ultrasound echo apparatus as discussed more fully hereinafter.

The method of ultrasonic imaging in which microbubbles formed by sonicating an aqueous protein solution are injected into a mammal to alter the acoustic properties of a predetermined area which is then ultrasonically scanned to obtain an image of the area for use in medical procedures is well known (e.g., see U.S. 4,572,203, U.S. 4,718,433 and U.S. 4,774,958, the contents of each of which are incorporated herein by reference). It is the use of the unique, stabilized perfluorocarbon-containing microbubbles of this invention which constitutes a novel improvement. Thus, in accordance with another embodiment of this invention, there is provided a method of ultrasonic imaging for use in medical procedures comprising the steps of forming an aqueous protein solution (e.g., aqueous dextrose albumin), subjecting said solution to high frequency sonication while exposed to perfluorocarbon gas, said sonication forming stabilized microbubbles of relatively uniform size, containing said perfluorocarbon,

and capable of transpulmonary passage, and using the stabilized microbubbles as an injectable contrast agent for said ultrasonic imaging.

Sonicated albumin has been used to study coronary flow reserve and immediate post-angioplasty anterograde blood flow reserve in humans. In humans without significant coronary artery disease, left main coronary artery injections of sonicated albumin before and after intracoronary papaverine result in time intensity curves which can be utilized to determine coronary flow reserve. It has been demonstrated that the washout of ultrasound contrast from the human myocardium in this setting correlates with coronary flow reserve measured by more invasive techniques.

Secondly, intracoronary sonicated albumin injections in humans with coronary artery disease, before and after angioplasty, has been done. The functional reserve of the myocardium supplied by the vessel undergoing angioplasty is immediately improved following angioplasty. The degree of improvement depends not on how visually successful the angioplasty was, but on how quantitatively successful the improvement in stenosis area was after angioplasty. Sonicated albumin does not reliably cross the pulmonary circulation into the left ventricular chamber following an intravenous injection, and thus at present cannot be used to non-invasively detect myocardial blood flow.

It has been observed that a microbubble radius decreases as a function of time in a still liquid due to a diffusion gradient present between the internal and external gases of the microbubble. An increase in microbubble size has a significant effect on the persistence of a microbubble within blood. The mean microbubble size of sonicated dextrose albumin ranges from between about 5 to about 6 microns. Since this size is ideal for transpulmonary passage, the major reason for the significant loss in left ventricular and myocardial videointensity produced following intravenous injection is

due to the significant diffusion of gases within the microbubble following intravenous injection during transit to the left ventricular cavity. Sonicated dextrose albumin enhanced with an inert gas such as perfluorocarbon, having a lower blood solubility than air, produces a significantly higher left ventricular and myocardial videointensity than sonicated albumin alone.

Because of high surface tension, the concentration of nitrogen and oxygen gas within the microbubble is much higher than that in blood, and thus there is a rapid diffusion of this gas into the blood stream following intravenous injection. Wible et al. (Circulation, 88:I-401, 1993) demonstrated that this diffusion process can be accelerated if one decreased the partial pressure of nitrogen within the blood stream by decreasing the inhaled fraction of nitrogen. This lower external concentration of nitrogen results in loss of the left ventricular videointensity produced by the same intravenous injection of sonicated albumin while inhaling room air. It has also been observed that oxygen rapidly diffuses out of gas bubbles into human blood (See Yang et al., J Biomech 3:275, 1971).

Both nitrogen and oxygen diffuse rapidly across these concentration gradients, but nitrogen appears to dissolve more slowly than oxygen into blood. Since nitrogen is the major component of air, decreasing the concentration gradient for nitrogen across the microbubble improves left ventricular and myocardial videointensity following intravenous injection. Exposing the microbubbles to a non-toxic gas having a lower blood solubility and/or microbubble diffusivity than that of nitrogen and having a gas density of greater than about .300 lb/ft^3 during sonication increases the size and stability of the microbubbles in sonicated dextrose albumin, while lowering the solubility and diffusivity of the microbubbles in blood.

The most preferred contrast agent of this invention is a perfluorocarbon-enhanced sonicated dextrose albumin solution comprised of a sonicated three-fold dilution of 5% human serum albumin with 5% dextrose. During sonication, said solution is perfused with perfluorocarbon for about 80 seconds, which lowers the solubility and diffusivity of the microbubble gas. The resulting microbubbles are concentrated at room temperature for at least about 120 ± 5 minutes, wherein the excess solution settles in the sonicating syringe. The excess solution is expelled and the concentrated microbubbles are transferred to a sterile syringe and injected intravenously into a mammal.

A second method or preparation includes hand agitatin 15 ± 2 ml of sonicated dextrose albumin with 8 ± 2 ml of a perfluorocarbon gas prior to sonication. Sonication then proceeds for 80 ± 5 seconds.

Using perfluorocarbon gas to enhance the sonicated contrast agent of this invention will result in a higher degree of myocardial opacification, endocardial border delineation, and enhanced detection of left-sided ultrasound Doppler signals, upon peripheral venous administration. Additionally, using perfluorocarbon gas during sonication creates a more stable microbubble concentration, which subsequently enables ultrasonic visualization of the liver and kidneys following an intravenous injection.

The following examples demonstrate the effect of inert gases on microbubble stability and diffusibility, and the effect of perfluoropropane and perfluorobutane-enhanced sonicated dextrose albumin on myocardial uptake and videointensity as well as on ultrasonic determination of renal perfusion. In all the following examples all parts and percentages are by weight, unless stated otherwise. All dilutions are by volume.

EXAMPLES

Preparation of Contrast Agents

Albumin (human) USP, 5% solution (hereinafter referred to as "albumin") and dextrose USP, 5% solution (hereinafter referred to as "dextrose") were obtained from a commercial source. The sonicating system used for sonication was a Heat System Ultrasonic Processor Model XL2020 (Heat Systems Inc., Farmingdale, New York). The 1/2 inch horn transducer was a resonating piezoelectric device. The 1/2 inch sonicating horn tip was sterilized prior to each sonication.

Sonication of Samples

Sixteen milliliter aliquots of albumin diluted 1:3 with dextrose were drawn up into a 35 cc "Monoject" syringe (Becton Dickinson and Company, Rutherford, NJ) and sonicated for 80 ± 1 seconds. The "Leur-Lok" of the 35 milliliter syringe was then attached to a stopcock. After mixing the dextrose albumin solution by hand for about 7 to about 10 seconds, the plunger was removed from the top of the syringe. The sterile sonicating horn was then lowered into the open end of the syringe until at the surface of the albumin-dextrose solution. The solution was placed at the horn tip and manually held at this position while continuously sonicating at a frequency of 20,000 Hz and a power output of 210 W for 80 ± 1 seconds to form a stable microbubble solution.

Gas Perfusion of Samples

During sonication, the dextrose albumin mixture was exposed to either perfluoropropane or perfluorobutane gas (Commercial Grade, 99.9% by weight). The gas was drawn up into a sterile syringe through a 0.22 μM filter (Micron Separations Inc., Westborough, Massachusetts) to prevent contamination. During sonication, 5 milliliters of perfluorocarbon gas was manually injected into the solution, over the 80 second time interval, through the stopcock so that the microbubbles produced contain this

less soluble gas. The total volume of perfluorocarbon-enhanced sonicated dextrose albumin produced with this formulation was 25±2 milliliters. These samples were then used for intravenous injection.

Microbubble Analysis

Microbubble size and purity was determined using hemocytometry. Microscopic inspection of the microbubbles was performed to determine if any coalescent microbubbles were present in the solution. Microbubble concentration was determined using a Coulter Counter. The contrast agent was rejected for use if any of the following conditions are present: the mean microbubble size was 4.0 to 6.0 microns; coalesced microbubbles or strands were detected by light microscopy; or the mean microbubble concentration was less than 0.8×10^9 or greater than 1.5×10^9 microbubble/milliliter. The sample was also rejected if the number of microbubbles greater than 10 microns in the sample was greater than 4%.

All samples were stored in 35 milliliter syringes until time of injection. All solutions were given within 36 hours of production. All samples were prepared in a laminar flow hood.

Preparation of Open-Chest Dogs

Mongrel dogs of either sex (15-30 kilograms) were anesthetized with sodium pentobarbital (30 milligram per kilogram intravenously), intubated, and ventilated initially with room air using a positive pressure respirator. A left thoracotomy was performed under sterile conditions and the pericardium incised. In addition to a 19 gauge peripheral intravenous line, eight French Catheters were placed in the femoral artery and vein for intravenous administration of ultrasound contrast agents and pressure monitoring. Through one femoral venous sheath, a 7F balloon-tipped thermodilution catheter was placed in the pulmonary artery using fluoroscopy for

determination of pulmonary artery pressure and cardiac output. A 7F pigtail catheter was introduced into the left ventricle for pressure measurements (left ventricular systolic and end-diastolic pressure) following injection of each ultrasound contrast agent.

Following adequate surgical exposure, a 3.5 Megahertz ultrasound transducer connected to a commercially available ultrasound scanner (Hewlett Packard Company; Andover, Massachusetts) was placed in a warm water bath. The bath overlays the anterior epicardial surface. The transducer was mounted on a clamp and lowered into the bath. It was adjusted until an optimal stable short axis view of the left and right ventricle had been obtained at the ventricular mid-papillary muscle level. These images could then be used to assess left ventricular cavity and myocardial uptake of contrast following intravenous injection.

EXAMPLE 1

Visually Apparent Consistent Myocardial Opacification with Perfluoropropane-Enhanced Sonicated Dextrose Albumin (PESDA)

Five open chest dogs were given incremental intravenous injections of perfluoropropane enhanced sonicated dextrose albumin (PESDA), produced as hereinbefore described, in doses of 0.02, 0.04, 0.06, 0.08 milliliter per kilogram (ml/kg). During intravenous injection, pulmonary artery pressure, left ventricular end-diastolic pressure, systolic pressure and cardiac output were monitored. Myocardial peak videointensity was measured using a 3.5 Megahertz epicardial transducer. Mean transit time of the contrast agent and half-time of contrast washout were also measured. Table 1 demonstrates that myocardial peal videointensity increased linearly with increasing dose of intravenous PESDA ($r = 0.65$, $p < 0.0001$).

TABLE 1

Dose ml/kg	PAP	LVSP	CO	MPVI
0.02	21.3 ± 3.8	105 ± 11.3	2.4 ± 0.5	7.6 ± 6.8
0.04	23.3 ± 4.6	103.5 ± 9.5	3.1 ± 0.9	17.9 ± 9.8
0.06	24.1 ± 4.0	102.7 ± 8.5	3.0 ± 0.9	22.2 ± 10.9
0.08	28.0 ± 3.5	102.2 ± 8.7	2.9 ± 0.8	25.5 ± 10.7

PAP = pulmonary artery pressure; LVSP = left ventricular systolic pressure; CO = cardiac output; MPVI = myocardial peak videointensity;

Visible myocardial opacification was seen in 100% of the 0.04 - 0.08 ml/kg intravenous injections. Table 1 demonstrates that low doses of PESDA produce consistent, visual myocardial opacification following intravenous injection; the degree of myocardial opacification is linearly related to the dosage; and, PESDA causes minimal hemodynamic changes and has physiologic washout times. PESDA is, therefore, a novel contrast agent which can non-invasively detect myocardial perfusion.

EXAMPLE 2

Use of PESDA to Quantify Coronary Blood Flow

Six open chest dogs were given 0.06 milliliter per kilogram (ml/kg) intravenous injections of perfluoropropane-enhanced sonicated dextrose albumin (PESDA), prepared as hereinbefore described. A total of 45 intravenous injections of PESDA were given in the eight dogs. Myocardial peak videointensity was measured and quantified using a 3.5 Megahertz epicardial transducer connected to a commercially available ultrasound scanner (Hewlett Packard Company, Andover, Massachusetts). Coronary blood flow was measured using a Transonic Doppler Flow Probe placed around the proximal left anterior descending artery. Cardiac output was measured using thermodilution. Table 2 demonstrates that there is a significant correlation between myocardial peak videointensity and coronary blood flow.

TABLE 2

Dog #	Dose	# Consecutive IV injections	Average MPVI	Average CBF (cc)	Average CO (L)
1	0.06 ml/kg	3	13	17	1.9
2	0.06 ml/kg	2	41	40	4.0
3	0.06 ml/kg	2	34	28	2.9
4	0.06 ml/kg	2	14	21	2.3
5	0.06 ml/kg	2	29	21	3.1
6	0.06 ml/kg	2	16	17	3.0

IV = intravenous; MPVI = myocardial peak videointensity; CBF = coronary blood flow; CO = cardiac output

Visually evident myocardial opacification was seen with PESDA following all intravenous injections. Multiple linear regression analysis demonstrated that MPVI correlated closest with coronary blood flow and not cardiac output. The myocardial PVI produced by intravenous injections of PESDA correlates with coronary blood flow over a wide range of flows and pathophysiologic events. This new ultrasound contrast agent, therefore, may be utilized to non-invasively quantify coronary blood flow in a wide variety of coronary diseases.

EXAMPLE 3

Use of PESDA to Non-Invasively Assess Renal Perfusion

Five dogs were given 0.06 milliliter per kilogram (ml/kg) intravenous injections of perfluoropropane-enhanced sonicated dextrose albumin (PESDA), produced as hereinbefore described. A total of 26 intravenous

injections were given. Renal imaging and qualitative contrast enhancement were performed during the intravenous injections using an external 4.5 Megahertz linear array transducer connected to a commercially available ultrasound scanner (Hewlett Packard company, Andover, Massachusetts). Renal artery blood flow was monitored using a Transonic Doppler probe around the renal artery. Ultrasound enhancement was qualitatively graded as "0" = no enhancement, "1+" = mild, "2+" = marked enhancement. Renal artery stenosis was induced at certain periods to decrease renal artery blood flow to less than 10% of baseline in order to determine a correlation between contrast and renal artery blood flow.

TABLE 3

Dog #	IV inject. dose	Average PRCV	Average RABF (ml)	Average PRCV following RAS	Qualitative enhancement
1	0.06 ml/kg	n/a	n/a	n/a	2+
2	0.06 ml/kg	15	n/a	n/a	2+
3	0.06 ml/kg	16	53	n/a	2+
4	0.06 ml/kg	28	117	9	2+
5	0.06 ml/kg	24	121	11	2+

IV = intravenous; PRCV = peak renal cortex videointensity; RABF = renal artery blood flow; RAS = renal artery stenosis; n/a = not available.

Following all 26 intravenous injections of PESDA, there was a 2+ contrast ultrasound enhancement of the renal cortex. The results in Table 3 demonstrate that renal artery and cortical blood flow abnormalities can be

detected using intravenous PESDA. These results also demonstrate that PESDA can be utilized to non-invasively detect renal artery stenosis or other causes of abnormal renal perfusion.

EXAMPLE 4

Use of PESDA to Visually Identify Acute Myocardial Ischemia and Reperfusion

Six open-chest dogs were given 0.06 milliliter per kilogram (ml/kg) intravenous injections of perfluoropropane-enhanced sonicated dextrose albumin, produced as hereinbefore described. Injections were given at baseline, within fifteen (15) minutes of ligation of the proximal left anterior descending artery (LAD), and after the LAD blood flow was restored. Ischemia was attained by ligating the LAD with silk or umbilical suture. The artery was clamped for a variable time interval and then released. The duration of ischemia was 10 minutes to 160 minutes. LAD blood flow was continuously monitored with a Transonic Doppler flow cuff. Myocardial peak videointensity (MPVI) was determined following each intravenous PESDA injection. Table 4 demonstrates that quantitatively evident contrast was seen in the anterior myocardium at baseline.

TABLE 4

Dog #	MPVI at Baseline	MPVI at Ligation	MPVI at Reperfusion
1	18	1.7	33
2	16	2.0	36
3	14	1.0	40
4	40	1.0	62
5	14	3.7	40
6	29	3.0	45

MPVI = myocardial peal videointensity

Table 4 demonstrates that intravenous PESDA can identify acutely ischemic and reperfused myocardium non-invasively. This new agent significantly improves the ability to rapidly identify whether coronary patency has

been achieved following mechanical or pharmacologic revascularization.

EXAMPLE 5

Use of Aminophylline to Enhance the Contrast Effects of PESDA

Six dogs were each given two equivalent quantities of two different samples of 0.08 milliliter per kilogram (ml/kg) intravenous injections of PESDA. One sample (PESDA-AM) was mixed with 2 milligrams (mg) of Aminophylline (AM) prior to sonication and another was sonicated without AM (PESDA). Myocardial peak videointensity was measured from the anterior myocardium using a 3.5 Megahertz epicardial transducer. Cardiac output was measured following each intravenous injection using thermodilution technique. Mean pulmonary and left ventricular systolic artery pressures were monitored during intravenous injection and coronary flow was measured using a Transonic Doppler flow cuff around the left anterior descending artery. Table 5 demonstrates the ability of this subtherapeutic dose of Aminophylline to enhance the contrast effects of PESDA.

TABLE 5

	CO	LAD CF	LVS	MPA	MPVI
PESDA alone	3.2±0.8 (L/min)	33±13 ml	103±11 (mm Hg)	24±5 (mm Hg)	21±12
PESDA - AM	3.5±1.0	34±18	104±11	24±5	30±12*

*p<0.0001 (paired t test)

CO = cardiac output; LAD CF = left anterior descending artery; LVS = left ventricular systolic pressure; MPA = mean pulmonary artery pressure; MPVI = myocardial peak videointensity; L/min. = liters per minute; mmHg = millimeters of mercury; ml = milliliter.

EXAMPLE 6

Safety and Efficacy of Perfluorobutane-Sonicated Dextrose Albumin (DF-SDA) in Dogs

Three open chest dogs were used to measure anterior and posterior myocardial peak videointensity produced by

either a 0.015 or 0.03 milliliter per kilogram intravenous injection of DF-SDA. These values were compared with the videointensity produced in these same regions by a 0.06 ml/kg intravenous injection of perfluoropropane-enhanced sonicated dextrose albumin (PESDA). Left ventricular and pulmonary artery pressures were measured before and after injection, as well as cardiac output.

The degree of left ventricular cavity shadowing was significantly less with DF-SDA than with PESDA in 5 dogs. This was verified in Table 5, where the peak posterior myocardial videointensity produced with DF-SDA was significantly higher than that produced by PESDA. This was entirely due to the significant decrease of left ventricular shadowing. As can be seen in Table 6, these doses of DF-SDA did not cause any change in pulmonary artery pressures or left ventricular pressures.

Table 5 - Peak Myocardial Videointensity of DF-SDA Compared with PESDA in Dogs

Contrast Agent	PVI (Anterior Wall)	PVI (Posterior Wall)
DF-SDA	2.8 ± 0.6 unit	2.5 ± 0.6 unit
PESDA	2.1 ± 0.4 unit	0.4 ± 0.3 unit

Table 6 - Effect of DF-SDA on Pressures

	Before Injection	After Injection
Mean Pulmonary Artery Pressure	15.5 ± 2.2mmHg	15.6 ± 2.9mmHg
Left Ventricular Systolic Pressure	93.5 ± 3.0mmHg	93.3 ± 5.4mmHg
Left Ventricular End-Diastolic Pressure	0.3 ± 0.7mmHg	0.3 ± 0.7mmHg

EXAMPLE 7

Stability of Perfluorobutane-
Sonicated Dextrose Albumin

The stability of DF-SDA over a 48-hour time period was also determined in three separate samples. Measurements of mean microbubble size, percentage of microbubbles above 10 microns, and mean microbubble concentration were performed immediately after production and again at 36 hours after production. Neither microbubble size nor concentration changed over this time period. These data are summarized in Table 7 below.

Table 7 - DF-SDA Stability

	Immediate	48 Hours
Mean size	4.7 ± 2.5μ	4.8 ± 2.7μ
Mean concentration	1.60 x 10^9	1.68 x 10^9
% Microbubbles > 10 microns	2.5%	3.6%

EXAMPLE 8

Perfluorobutane-Sonicated Dextrose
Albumin Effects on Human Blood

Eight blood samples from four human volunteers were obtained to assess the effect of perfluorobutane gas on white and red blood cell counts. Each person had one sample exposed to 2 milliliters of perfluorobutane and one of their samples exposed to room air. As can be seen, there was no effect of perfluorobutane on white or red blood cell count, platelet count, or number of abnormal red blood cells seen on the peripheral blood smear (Table 8). (Results are the average of four volunteers' blood samples.)

Table 8 - Effect of Perfluorobutane on Blood

	Before Perfluorobutane	After Perfluorobutane
White blood count	6.7×10^3	6.6×10^3
Red blood count	4.8×10^6	4.8×10^6
Platelet count	Adequate	Adequate

Therefore, perfluorobutane does not appear to adversely affect blood or sonicated albumin.

These preliminary data in animals and in-vitro studies with human blood demonstrate the safety of intravenous DF-SDA. It is critical to study the safety of DF-SDA in patients with coronary artery disease during resting and stress echocardiography. Perfluorobutane has already been used safely in humans during intraocular surgery and we anticipate from this information and our preliminary data that it will be safe in humans.

EXAMPLE 9

Perfluoropentane-Sonicated Dextrose Albumin (P5SDA)

Myocardial contrast from an intravenous (IV) injection of perfluoropropane (188 grams/mole)-exposed sonicated dextrose albumin (PESDA) microbubbles is limited in detecting posterolateral perfusion abnormalities because of attenuation produced by microbubbles within the left ventricular (LV) cavity. Since the mechanism of improved contrast with these agents is related to gas diffusivity, it was hypothesized that incorporating even higher molecular weight gases like perfluoropentane (288 grams/mole) into sonicated dextrose albumin (C5SDA) would permit even smaller IV quantities to be given, preventing cavity attenuation and improving the detection of posterior perfusion abnormalities. Accordingly, the anterior and posterior peak myocardial videointensity (PMVI) following

IV injections of 0.06 ml/kg PESDA versus 0.015-0.030 ml/kg IV injections of C5SDA was compared in seven open chest dogs. Injections were given under baseline conditions, during acute ischemia produced by adenosine stress or left circumflex artery (LCX) ligation, and during reperfusion of the occluded vessel.

In six of the seven dogs prior to ischemia and during reperfusion, the lower doses of IV C5SDA produced a visually evident improvement in posterior myocardial contrast compared to PESDA, and a higher PMVI (3.1±2.4 Units C5SDA versus 0.7±1.2 Units PESDA; $p<0.0001$). Despite the lower dose of C5SDA, there were no differences in anterior PMVI (4.4±2.0 Units PESDA versus 4.3±2.4 Units (C5SDA).

There were no significant LV or pulmonary artery pressure changes following IV C5SDA for continuous ultrasound imaging, in doses of 0.015 - 0.03 ml/kg, and no change in cardiac output.

EXAMPLE 10

Use of Perfluoropropane and Perfluorobutane Enhanced Sonicated Dextrose Albumin to Determine Myocardial Blood Flow in Humans

For humans, doses of from about as small as .0025 up to .08 ml/kg are given depending on the ultrasonic procedure used. The contrast agent is given by peripheral intravenous injection over 3-5 seconds followed by a 10 milliliter normal saline flush. (The dose range is patient-specific: large patients may require slightly higher doses to produce equivalent left ventricular contrast effects). Generally a patient would receive a 0.01 ml/kg of perfluoropropane sonicated dextrose albuminor 0.0015 ml/kg perfluorobutane sonicated dextrose albumin, as the initial injection. If this fails to produce myocardial opacification, the dose would then be doubled. The dose which produces myocardial opacification and improved detection of abnormal wall motion and left

ventricular ejection fraction is used to determine myocardial blood flow by contrast echocardiography performed using the standard technique as described in Weyman, Arthur E., "Principles and Practice of Echocardiography", Lea & Febiger, Malvern, PA (1994, 2d Edition) and using the commercially available Hewlett Packard Sonos 1500 Phased Array Imaging System (Hewlett Packard, Andover, Massachusetts). Throughout the echocardiographic procedure, the patient's heart rate, blood pressure and oxygen saturation are monitored and recorded. The peak videointensity (corrected for baseline intensity) in the left ventricular cavity and myocardium for each injection is obtained.

Myocardial contrast produced from intravenous injection of two different molecular weight perfluorocarbon containing microbubbles in humans. The peak anterior myocardial videointensity (PMVI) and duration of acoustic shadowing (AS) produced by up to 0.02 ml/kg intravenous injection of perfluoropropane (molecular weight 188 grams per mole (g/m)) exposed sonicated dextrose albumin (C3SDA) were compared with 0.007 ml/kg intravenous injection of perfluorobutane exposed sonicated dextrose albumin (C4SDA) in 24 patients.

PCMB	N	Dose	PMVI	AS (Sec)	% 1-2+MC
C3SDA	14	.02±.08	2.1±1.5	50±15	5 (36%)
C4SDA	10	.007±0.01*	1.9±1.8	31±21*	8 (80%)

*p<0.05 compared to C3SDA

Despite lowering the dose, intravenous injection of C4SDA produced significantly higher myocardial contrast, with significantly less AS. Thus intravenous perfluoropropane and perfluorobutane exposed sonicated dextrose albumin can be used safely in humans with significantly better myocardial contrast for perfluorobutane than for lower molecular weight perfluoropropane.

As can be seen from the foregoing, the invention accomplishes at least all of its objectives.

What is claimed is:

1. A pharmaceutically acceptable ultrasound contrast agent which relies on microbubbles for echogenicity, said agent comprising: microbubbles with an internal atmosphere enhanced with perfluorocarbon gas.

2. The contrast agent of claim 1 wherein said perfluorocarbon gas is of the following formula:

wherein n is 1-10

3. The contrast agent of claim 2 wherein n is 4 and said perfluorocarbon gas is perfluorobutane.

4. The contrast agent is claim 2 wherein n is 5 and said perfluorocarbon gas is perfluoropropane.

5. The contrast agent of claim 1 further comprising human serum albumin for coating said microbubbles.

6. The contrast agent of claim 5 wherein said human serum albumin is diluted 2 to 8 fold with dextrose.

7. The contrast agent of claim 6 wherein said human serum albumin is a 5% by weight solution.

8. The method of claim 6 wherein said dextrose is a 5% by weight solution.

9. An ultrasound contrast agent comprising a sonicated aqueous albumin-dextrose solution comprising: between about a two-fold and about an eight-fold dilution of albumin with between about 5% to about 50% by weight

dextrose; said albumin between about 2% to about 10% by weight human serum albumin; and microbubbles the gaseous content of which contain an amount of perfluorocarbon gas which is effective for visually detecting myocardial perfusion upon echocardiogram following peripheral intravenous injection of said agent into a host.

10. The contrast agent of claim 9 wherein said dilution of albumin with dextrose is a 3-fold dilution.

11. The contrast agent of claim 9 wherein said human serum albumin is a 5% by weight solution.

12. The contrast agent of claim 9 wherein said dextrose is a 5% by weight solution.

13. A method for myocardial, renal or hepatic opacification comprising the steps of: (a)obtaining an echo contrast agent which comprises: (i) an aqueous albumin-dextrose solution containing between about a two-fold and about an eight-fold dilution of between about 5% to about 50% by weight dextrose and between about 2% to about 10% by weight human serum albumin, and (ii) microbubbles the gaseous content of which contain an amount of perfluorocarbon gas which is effective for visually detecting myocardial perfusion by echocardiogram following peripheral intravenous injection of said agent into a host; (b) introducing said echo contrast agent into a host by intravenous injection; and (c) performing an echo contrast study on said host using a suitable Doppler or ultrasound echo apparatus.

14. A method of ultrasonic imaging for use in medical procedures, comprising the steps of: (a) intravenously injecting perfluorocarbon-containing microbubbles into a mammal to thereby alter the acoustic properties of a predetermined area, and (b) ultrasonically scanning an area

including said predetermined area so as to obtain an image of said predetermined area.

15. The method of claim 13 wherein said step of obtaining said echo contrast agent further comprises the step of: forming an aqueous solution of human serum albumin and dextrose.

16. The method of claim 15 wherein said solution is one part human serum albumin to 3 parts dextrose.

17. The method of claim 15 further comprising the step of: sonicating said solution to produce gas-filled microbubbles.

18. The method of claim 17 further comprising the step of: perfusing said solution with perfluorocarbon gas during sonication.

19. The method of claim 18 wherein said perfluorocarbon gas is selected from the group consisting of perfluoromethane, perfluoroethane, perfluoropropane, perfluorobutane and perfluoropentane.

20. A pharmaceutically acceptable contrast agent which relies on microbubbles for echogenicity, said agent comprising: microbubbles with an interior atmosphere enhanced with perfluorobutane gas.

INTERNATIONAL SEARCH REPORT

Inter tional Application No
PCT/US 96/05468

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K49/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 93 05819 A (QUAY STEVEN C) 1 April 1993 see abstract see tables 2,3 see examples 1-3,6 see claim 11	1,2,5-19 1-20

X	J AM COLL CARDIOL, FEB 1995, VOL. 25, NO. 2, PAGE(S) 509-15, XP000590866 PORTER TR ET AL: "Visually discernible myocardial echocardiographic contrast after intravenous injection of sonicated dextrose albumin microbubbles containing high molecular weight, less soluble gases." see abstract see paragraph: discussion	1-20

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 October 1996	22.10.96
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	**Authorized officer** Dullaart, A

Form PCT/ISA/210 (second sheet) (July 1992)

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	67TH SCIENTIFIC SESSIONS OF THE AMERICAN HEART ASSOCIATION, DALLAS, TEXAS, USA, NOVEMBER 14-17, 1994. IN: CIRCULATION, 1994, VOL. 90, NO. 4 PART 2, PAGE(S) I69, XP000577055 XIE F ET AL: "Perfluoropropane enhanced sonicated dextrose albumin produces visually apparent consistent myocardial opacification with physiologic washout and minimal hemodynamic changes following venous injection" see abstract number 362 ---	1-20
Y	J AM SOC ECHOCARDIOGR, SEP-OCT 1994, VOL. 7, NO. 5, PAGE(S) 465-71, XP000590864 PORTER TR ET AL: "Multifold sonicated dilutions of albumin with fifty percent dextrose improve left ventricular contrast videointensity after intravenous injection in human beings." see abstract see paragraph: discussion ---	1-20
X	44TH ANNUAL SCIENTIFIC SESSION OF THE AMERICAN COLLEGE OF CARDIOLOGY, NEW ORLEANS, LOUISIANA, USA, MARCH 19-22, 1995. IN: JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY, SPEC. ISSUE, PAGE(S) 38A, XP000577057 KRICSFELD A ET AL: "Detection of regional perfusion abnormalities during adenosine stress echocardiography using intravenous perfluoropropane-enhanced sonicated dextrose albumin" see abstract number 703-2 ---	1-20
X	44TH ANNUAL SCIENTIFIC SESSION OF THE AMERICAN COLLEGE OF CARDIOLOGY, NEW ORLEANS, LOUISIANA, USA, MARCH 19-22, 1995. IN: JOURNAL OF THE AMERICAN COLLEGE OF CARDIOLOGY, SPEC. ISSUE, PAGE(S) 205A., XP000577058 PORTER T ET AL: "Echocardiographic detection of residual coronary flow abnormalities and stenosis severity after coronary reperfusion using intravenous perfluoropropane-enhanced sonicated dextrose albumin" see abstract number 955-60 ---	1-20

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	67TH SCIENTIFIC SESSIONS OF THE AMERICAN HEART ASSOCIATION, DALLAS, TEXAS, USA, NOVEMBER 14-17, 1994. CIRCULATION, 1994, VOL. 90, NO. 4 PART 2, PAGE(S) I555, XP000577054 XIE F ET AL: "Acute myocardial ischemia and reperfusion can be visually identified non-invasively with intravenous perfluoropropane-enhanced sonicated dextrose albumin ultrasound contrast" see abstract number 2989 ---	1-20
P,X	INT J CARD IMAGING, JUN 1995, VOL. 11, NO. 2, PAGE(S) 117-25, XP000590839 PORTER TR ET AL: "The mechanism and clinical implication of improved left ventricular videointensity following intravenous injection of multi-fold dilutions of albumin with dextrose." see abstract see paragraph: Results ---	1-20
P,X	J AM COLL CARDIOL, JUL 1995, VOL. 26, NO. 1, PAGE(S) 33-40, XP000590865 PORTER TR ET AL: "Noninvasive identification of acute myocardial ischemia and reperfusion with contrast ultrasound using intravenous perfluoropropane -exposed sonicated dextrose albumin." see abstract see paragraph: Discussion ---	1-20
P,X	WO 95 23615 A (HOLMES MICHAEL JOHN ;NYCOMED IMAGING AS (NO); KLAVENESS JO (NO); B) 8 September 1995 see abstract see examples 3,4 -----	1,2,5-19

INTERNATIONAL SEARCH REPORT

In ational application No.
PCT/US96/05468

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 13 - 19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 1-2, 5-18
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Please see annex

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

In view of the large number of compounds, which are defined by the general definitions used in the independent claims, the search had to be restricted for economic reasons. The search was limited to the compounds for which pharmacological data was given and/or the compounds mentioned in the claims, and to the general idea underlying the application (see Guidelines, chapter III, paragraph 2.3)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inv tional Application No
PCT/US 96/05468

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9305819	01-04-93	US-A-	5409688	25-04-95
		AU-A-	2550392	27-04-93
		CA-A-	2119129	01-04-93
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		CZ-A-	9400612	19-10-94
		EP-A-	0605477	13-07-94
		FI-A-	941242	16-05-94
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		US-A-	5393524	28-02-95
		US-A-	5558854	24-09-96
		US-A-	5558094	24-09-96
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WO-A-9523615	08-09-95	AU-A-	1818695	18-09-95